Developmental Cell, Vol. 8, 677–688, May, 2005, Copyright ©2005 by Elsevier Inc. DOI 10.1016/j.devcel.2005.02.019

Analysis of Notch Function in Presomitic Mesoderm Suggests a γ-Secretase-Independent Role for Presenilins in Somite Differentiation

Stacey S. Huppert,[1] Ma. Xenia G. Ilagan,[1] Bart De Strooper,[2] and Raphael Kopan[1,*]

[1]Department of Molecular Biology and Pharmacology
Department of Medicine
Division of Dermatology
Washington University School of Medicine
St. Louis, Missouri 63110

[2]Center for Human Genetics
KULeuven and Flanders Interuniversity Institute for Biotechnology (VIB)
3000 Leuven
Belgium

Summary

The role of Notch signaling in general and presenilin in particular was analyzed during mouse somitogenesis. We visualize cyclical production of activated Notch (NICD) and establish that somitogenesis requires less NICD than any other tissue in early mouse embryos. Indeed, formation of cervical somites proceeds in *Notch1; Notch2*-deficient embryos. This is in contrast to mice lacking all *presenilin* alleles, which have no somites. Since *Nicastrin*-, Pen-2-, and APH-1a-deficient embryos have anterior somites without γ-secretase, *presenilin* may have a γ-secretase-independent role in somitogenesis. Embryos triple homozygous for both *presenilin* null alleles and a Notch allele that is a poor substrate for presenilin ($N1^{V\rightarrow G}$) experience fortuitous cleavage of $N1^{V\rightarrow G}$ by another protease. This restores NICD, anterior segmentation, and bilateral symmetry but does not rescue rostral/caudal identities. These data clarify multiple roles for Notch signaling during segmentation and suggest that the earliest stages of somitogenesis are regulated by both Notch-dependent and Notch-independent functions of presenilin.

Introduction

The *Notch* genes encode single-pass type I transmembrane receptors that transduce extracellular signals during multiple steps of metazoan development. It is widely accepted that Notch receptors are activated through ligand-regulated proteolysis and shedding of the extracellular domain. This triggers a γ-secretase-dependent proteolytic release of the Notch intracellular domain (NICD) from the membrane, permitting NICD to form a nuclear complex with CBF1/Su(H)/Lag1 (CSL) to activate transcription of downstream targets. Genetic evidence in *Drosophila* suggests that an alternative, CSL-independent signaling mechanism exists (Arias et al., 2002; Brennan and Gardner, 2002), although it is unclear what the actual mechanism is and whether proteolysis is required.

In somitogenesis, γ-secretase-dependent proteolysis and NICD/CSL-dependent transcription are thought to act as a component of the segmentation clock (Aulehla and Herrmann, 2004; Bessho and Kageyama, 2003; Giudicelli and Lewis, 2004; Pourquie, 2004; Rida et al., 2004). Several experimental observations suggest that Notch signaling acts at two additional steps: establishment of rostral/caudal (R/C) identities and enabling cleft formation. The involvement of Notch in the segmentation clock is predicted by the phenotypes associated with Notch pathway mutants and the cyclic expression of Notch target genes, implying that Notch signaling is both a readout and a component of the clock. Studies concerning the target genes *Lfng* (Dale et al., 2003), *Hes1* (Hirata et al., 2002), and *Hes7* (Hirata et al., 2004) suggest that the oscillations of the clock are achieved through negative feedback loops in which Notch targets inhibit Notch activation, transcriptional output, or both. Modeling the clock mechanism based on observations made in zebrafish suggest that the clock is insensitive to the amplitude of Notch activation (and thus to the amount of NICD [Giudicelli and Lewis, 2004]); however, the actual level of Notch activity required for segmentation has not been demonstrated.

The role of Notch signaling in establishment of R/C identity was investigated in mouse and zebrafish mutants (Sawada et al., 2000; Takahashi et al., 2000; Takahashi et al., 2003). In the anterior presomitic mesoderm, the Notch pathway forms a complicated regulatory loop with one of its targets, *Mesp2*, and its ligands, *Delta1* and *Delta3* (Takahashi et al., 2003). When either Mesp2 or Dll1 protein is lost, the resulting phenotype is of a single identity, either caudal or rostral, respectively (A. Gossler, personal communication; Bussen et al., 2004; Takahashi et al., 2000). Thus, Notch-mediated transcription is involved in the initiation of distinct R/C identities. The second step of R/C identity requires segregation of R/C identities from each other and maintenance of the separated cohorts of cells (Kulesa and Fraser, 2002). *Dll3*, *Lfng*, and *Paraxis* appear to be involved in this step, as mutant cells acquire stable R/C identities but fail to segregate into distant domains (Johnson et al., 2001; Zhang et al., 2002). Some models suggest that the cells of the presomitic mesoderm (PSM) synchronously oscillate between rostral and caudal cell states, and once stabilized and segregated, a boundary forms at the juxtaposition of rostral and caudal cells (Meinhardt, 1986; Pourquie, 2001). One of these boundaries forms the cleft separating the somites, and the other will form a permanent divide during the process of resegmentation (i.e., the process that culminates in the formation of the vertebra from the caudal compartment and anterior compartment of the adjacent somites) (Aoyama and Asamoto, 2000). However, segregation of R/C fates is not required for clefting: alleles of *paraxis* (Johnson et al., 2001) and *Mesp2* (Nomura-Kitabayashi et al., 2002), which do not have a true juxtaposition of cellular cohorts with R/C identities, are able to form clefts between somites. Clefting may thus be attributed to Notch activity: expression of activated Notch can induce an ectopic cleft

*Correspondence: kopan@wustl.edu

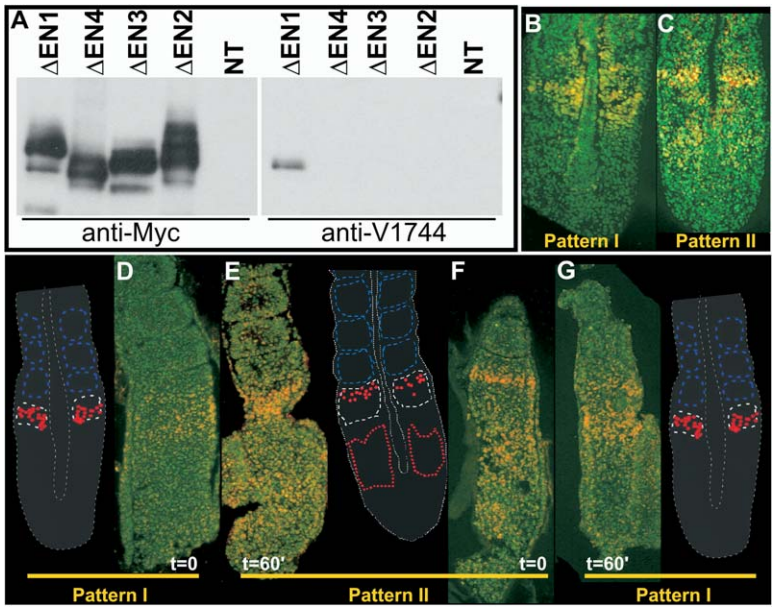

Figure 1. Notch Undergoes Cyclic Proteolysis in the PSM

(A) Western analysis showing the specificity of the Val1744 antibody for Notch1 NICD. Myc-tagged constructs that mimic the ligand-activated form of all four Notch's (ΔEN1-4) were transfected into 293T cells and assayed for their presence with anti-Myc (9E10) and the specificity for NICD detection (anti-Val1744). Only NICD1 was detected.

(B–G) Immunofluorescence of E10.5 PSM sections, nuclei (green), NICD (red), and overlapping NICD in nuclei (yellow).

(B) One representative section of serially sectioned PSMs stained with V1744 antibodies was traced to generate a schematic representing the observed staining patterns. Nuclear NICD stain was traced in red and somite boundaries traced in blue. Somite identity was determined after viewing the entire series as a movie loop and single images (available upon request). Pattern I: NICD accumulation in a broad area around somite S-1 and S0, observed in 3 of 14 PSM.

(C) Pattern II, accumulation of NICD in a tight, anterior region coincident with the cleft-forming area between S-1 and S0, and a second diffuse area of NICD accumulation in the middle of the PSM, observed in 11 of 14 PSM.

(D–G) Representative pairs ([D and E] and [F and G]) of half PSM cultures, either before (D and F) or after (E and G) 60 min incubation. In the half PSM culture experiments, we were able to observe changes from Pattern I to II in four PSM pairs. Of 12 cultured pairs, 5 produced complete serial sections that were successfully stained. Four pairs shifted from one pattern to the next. In one pair, we were not able to see a significant change from Pattern I to describe the cultured half as a Pattern II.

in competent PSM tissue (Sato et al., 2002; Serth et al., 2003). These experiments showed that Notch activation was sufficient, but did not demonstrate that activation of Notch signaling was also necessary for cleft formation. Importantly, Notch pathway mutants in both mouse (*RBP-Jκ*, in which no canonical Notch signal is produced [Oka et al., 1995]) and zebrafish (*notch1a* or *deadly seven* [Holley et al., 2002] and *deltaD* or *after eight* [Holley et al., 2000]) are still able to form anterior somites. This suggests that somitogenesis has fail-safe mechanisms, perhaps through redundancy of clocks maintained by multiple pathways, to ensure accurate conformity.

The identification of the epitope generated by γ-secretase cleavage (Schroeter et al., 1998) allowed us to identify mutations that diminish Notch proteolysis. We created a mouse in which we knock in one such mutation, changing the codon GTG (valine) to GGG (glycine) at position 1744 of Notch1. The $N1^{V\rightarrow G}$ allele allowed us to establish that NICD production was required for Notch signaling (Huppert et al., 2000). Analysis of $N1^{V\rightarrow G}$ homozygous embryos (henceforth $N1^{V\rightarrow G}$–/–) demonstrated unequivocally that efficient Notch1 intracellular processing is required for embryonic viability, neurogenesis, and vascular development because $N1^{V\rightarrow G}$–/– embryos have identical disruptions in these processes compared to the *Notch1* null embryos ($N1^{\Delta 1}$ and $N1^{in32}$; Conlon et al., 1995; Huppert et al., 2000; Swiatek et al., 1994). The one exception was somitogenesis, which proceeded normally in $N1^{V\rightarrow G}$–/– embryos (Huppert et al., 2000). This observation suggests that somitogenesis may indeed progress normally with vanishing amounts of NICD, which are below the threshold in any other tissue, and is consistent with recent mathematical models proposing that the functional threshold required for NICD to maintain synchronicity in the somite is low (Lewis, 2003). However, an apparent paradox emerges when one considers that embryos homozygous for a hypomorphic presenilin1 (PS1) allele have highly penetrant segmentation defects but no significant impact on other tissues (Rozmahel et al., 2002b). This result indicates that the PSM requires the *highest* amounts of presenilin.

In this manuscript, we present several lines of evidence to resolve this paradox by demonstrating that while the threshold for NICD activity in the PSM is the *lowest* in the embryo, a limiting role for presenilin does exist and appears to be independent of γ-secretase.

Results

Notch1 Undergoes Cyclical Intramembranous Proteolysis in the PSM

To provide a framework on which to begin further investigations of the mechanisms by which Notch contributes to somitogenesis, we first wanted to determine if Notch1 is activated via proteolysis in this context. To monitor Notch1 proteolysis, we used an antibody specific for the neo-epitope generated by γ-secretase cleavage of Notch1 (Val1744; Figure 1A). This antibody is specific for the NICD form of Notch1 and does not recognize the epitope in the linear substrate. Notch4, also cleaved at a valine (Saxena et al., 2001), and the NICDs produced from other Notch proteins are not recognized by Val1744 (Figure 1A).

Models proposing Notch function during segmentation (Aulehla and Herrmann, 2004; Bessho and Kageyama, 2003; Giudicelli and Lewis, 2004; Pourquie, 2003;

Rida et al., 2004) predict that we should observe NICD at the anterior of the PSM where a somite cleft is formed between somite S-1 and S0 (Sato et al., 2002). We would also expect NICD to be detected in the region where R/C identities are being established within somitic region II (Saga and Takeda, 2001). More posterior, NICD should cycle as do its target genes (Lfng, Hes1, Hes7, and Hey2) in region I (Aulehla and Johnson, 1999; Bessho et al., 2001; Forsberg et al., 1998; Jouve et al., 2000; Leimeister et al., 2000; Saga and Takeda, 2001). Finally, NICD should be localized periodically to the caudal neuropore, where cycling is initiated (Dale et al., 2003). To visualize these possible dynamic patterns of NICD, we sectioned and analyzed the entire PSM from 14 E10.5 embryos and examined NICD localization. Two patterns were discernable (Figures 1B and 1C). Pattern I (observed in 3 of 14 stained PSMs) was characterized by NICD accumulation in a broad area around somite S-1 and S0. The more abundant Pattern II (observed in 11 of 14 stained PSMs) is defined by the accumulation of NICD in a tighter, anterior region coincident with the cleft-forming area between S-1 and S0, and a second diffuse area of NICD accumulation in the middle of the PSM. These patterns are suggestive of cyclic activation of Notch1. We did not detect a posterior signal in region I or at the caudal neuropore. To directly determine if NICD/Notch activation cycles, as do its target genes, we isolated and dissected the PSM of E10.5 embryos, fixed one half (time zero), and cultured the other half for 60 min (Correia and Conlon, 2000). We observed a change in the Pattern from I to II (Figures 1D and 1E) and vice versa (Figures 1F and 1G). These results clearly demonstrate that NICD, and thus Notch activation, is cycling and that a third pattern element is either too transient (or at this level of resolution, too difficult to distinguish from Pattern II) or nonexistent.

Two opposing hypotheses could explain the lack of NICD in the posterior region. If the amplitude of Notch activation grows as the activation wave moves to a more anterior position (as predicted in Aulehla et al., 2003), one would expect that the amounts of NICD in the most posterior region would be too low for the Val1744 antibody to detect. Alternatively, NICD may not be required in the posterior region to initiate the cycling; either initiation is under Wnt control (Aulehla and Herrmann, 2004; Aulehla et al., 2003; Galceran et al., 2004; Hofmann et al., 2004) or the self-repressing Hes1 (Hirata et al., 2002) and Hes7 (Hirata et al., 2004) regulate their own expression via a negative feedback loop. While our data cannot unequivocally rule out a model in which Notch1 initiates the cycle, it clearly supports the notion that Notch activation can reinforce/refine the pattern.

Threshold Requirement for Notch Activation in PSM

Since our results above show that NICD is produced and cycles in the PSM, yet no segmentation phenotype is observed in $N1^{V\rightarrow G}$–/– embryos, we examined whether the threshold for Notch activity in the PSM for normal segmentation is lower than in other tissues. We first took advantage of the hypomorphic residual processing nature of the $N1^{V\rightarrow G}$ allele and the partially penetrant segmentation phenotype of Dll1 heterozygous animals. *Dll1*–/+ animals exhibit a kink in their tails (Cordes et al., 2004) that is dramatically increased in compound heterozygote *Dll1*–/+;$N1^{V\rightarrow G}$–/+ animals (7%, 2 out of 28 in *Dll1* heterozygotes; 53%, 10 out of 19 in compound heterozygotes, see Supplemental Figure S2B, inset, available with this article online). These data show that the threshold of Notch activity required for segmentation changes with time (and only hence with position), increasing as the tailbud/PSM matures and generates more posterior (tail) structures.

To further examine if functional reduction in ligand can trigger the $N1^{V\rightarrow G}$ homozygous embryos to reveal a segmentation phenotype, we examined whether the embryonic defects of $N1^{V\rightarrow G}$–/– were enhanced by reduction in the dosage of *Delta1*. The neural tube appears more severely kinked in *Dll1*–/+;$N1^{V\rightarrow G}$–/– compared to $N1^{V\rightarrow G}$–/– and both genotypes are improperly expressing *Uncx4.1* in the neural tube (Neidhardt et al., 1997; Figures 2B and 2D). However, segmentation is only slightly impacted in *Dll1*–/+;$N1^{V\rightarrow G}$–/– compared to $N1^{V\rightarrow G}$–/– in both anterior and posterior (Figures 2A and 2C, embryos processed in the same tube): *Dll1*–/+;$N1^{V\rightarrow G}$–/– embryos maintain bilateral symmetry, but the caudal compartment size, as reflected by the marker *Uncx4.1* (Figure 2C), is mildly reduced (Neidhardt et al., 1997). These data inform us that anterior and trunk segmentation is not as sensitive to a reduction in Notch activity as neural tissue.

If segmentation was independent of NICD but required Notch protein, it will proceed unimpeded in the absence of NICD. However, in a tissue that is relatively insensitive to NICD amounts, but requires some NICD nonetheless, reduction of NICD will eventually produce a segmentation phenotype. Using the $N1^{V\rightarrow G}$ allele allowed us to start with NICD at a level that is sufficient for the process of segmentation (Huppert et al., 2000). We then titrated out the amount of presenilin, the catalytic component of γ-secretase, genetically. Theoretically, this should eventually lower the amount of NICD produced as the presenilin dosage was lowered. We hypothesized that at the point $N1^{V\rightarrow G}$ proteolysis becomes limiting, a segmentation phenotype will be detected. If NICD was dispensable for Notch activity in the PSM, no phenotype will be detected.

Animals lacking *presenilin2* (*PS2*) are viable with no overt phenotypes (Donoviel et al., 1999; Herreman et al., 1999; Steiner et al., 1999). Loss of *PS1* produced embryonic phenotypes, including defects in somitogenesis (Koizumi et al., 2001; Shen et al., 1997; Wong et al., 1997). We therefore chose to start the analysis by reducing the dose of *PS1*. As would be expected if $N1^{V\rightarrow G}$ function depended on producing limiting amounts of NICD, loss of one allele of *PS1* (*PS1*–/+) altered the phenotype of $N1^{V\rightarrow G}$–/– embryos (Figure 3). Expression of *Delta1* in the neural tube (Bettenhausen et al., 1995) was increased (Figure 3D), a sign of premature differentiation (de la Pompa et al., 1997), and an enhancement of the neural phenotype associated with $N1^{V\rightarrow G}$–/– embryos was observed (Huppert et al., 2000). *Uncx4.1* staining, a marker for the caudal compartment, was slightly reduced, suggesting that fewer cells committed to the caudal fate (compare Figure 3B to 2A and Huppert et al., 2000). Although these embryos are develop-

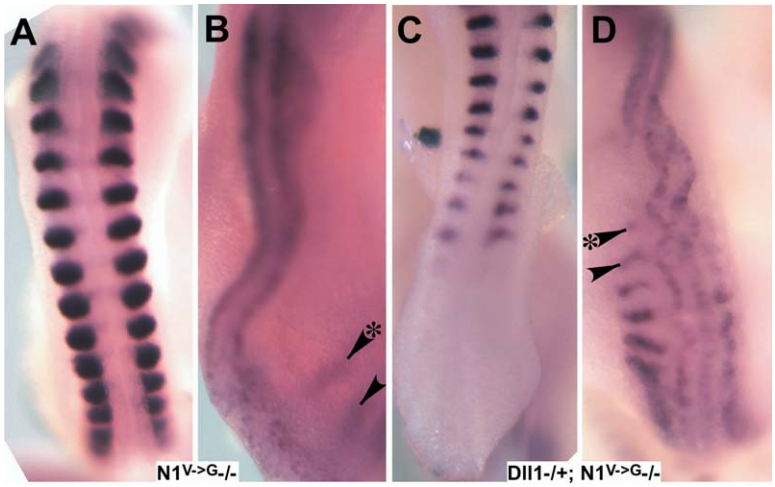

Figure 2. Neural Tissue Is More Sensitive than PSM to the Level of Notch Activation

Whole-mount in situ hybridizations for *Uncx4.1* of E9.5 to E10 embryos. Posterior down. Posterior views in (A) and (C) and anterior views in (B) and (D). Two different $N1^{V\rightarrow G}$–/– embryos shown in (A) and (B). Two different *Dll1–/+;*$N1^{V\rightarrow G}$–/– embryos shown in (C) and (D). Enhanced kinked neural tube (D) and slightly less *Uncx4.1* expression (C) is observed in *Dll1–/+;*$N1^{V\rightarrow G}$–/–. Arrowheads (B and D) point to normal *Uncx4.1* expression in the anterior somites. Overall, neurogenesis is affected more than somitogenesis (anterior and posterior). Minor developmental retardation in *Dll1–/+;*$N1^{V\rightarrow G}$–/– embryos is observed.

mentally retarded, the segmentation phenotype described here was not as severe as in $N1^{\Delta 1}$–/– embryos (bilateral symmetry not maintained) (Conlon et al., 1995; Swiatek et al., 1994), again demonstrating that neurogenesis requires a higher level of Notch activation relative to somitogenesis.

To determine if further reduction in *presenilin* will phenocopy $N^{\Delta 1}$–/– in the $N1^{V\rightarrow G}$–/– PSM, we examined the compound effect of reducing *PS2* dosage in *PS1–/+;* $N1^{V\rightarrow G}$–/– embryos. As a control, we analyzed the same *presenilin* dosage in the background of $N1^{\Delta 1}$–/– embryos. Removing *presenilin* alleles does not further modify the Notch1 null phenotype: (*PS1–/+;PS2–/+;* $N1^{\Delta 1}$–/–) and (*PS1–/+;PS2–/–;*$N1^{\Delta 1}$–/–) embryos were similar in size and phenotype to the null $N1^{\Delta 1}$–/– animals (Figure 3H; Supplemental Figure S3B; Conlon et al., 1995; Huppert et al., 2000). *Uncx4.1* shows lack of consistent R/C compartments and reduced bilateral symmetry. In contrast, the *PS1–/+;PS2–/+;*$N1^{V\rightarrow G}$–/– embryos display enhanced developmental retardation as would be expected if the level of NICD is important (Figure 3F). However, even these embryos show some commitment to the caudal fate as illustrated by *Uncx4.1* expression in some posterior somites (Figure 3F′). Importantly, *PS1–/+;PS2–/+;*$N1^{V\rightarrow G}$ embryos still formed clefts.

As described previously (Donoviel et al., 1999; Herreman et al., 1999), the phenotype of *PS1–/–;PS2–/–* embryos is more severe than loss of *PS1–/–* alone; *PS1–/–; PS2–/–* embryos do not form somites (Donoviel et al., 1999). We collected triple homozygous embryos *PS1–/–; PS2–/–;*$N1^{V\rightarrow G}$ (observed 6/461 embryos from various parental genotypes, expected 10/461; Figure 4H) and *PS1–/–;PS2–/–;*$N1^{\Delta 1}$–/– (observed 1/105 embryos from various parental genotypes, expected 3/105; Figure 4G). Surprisingly, morphological examination revealed the presence of somites in *PS1–/–;PS2–/–;*$N1^{V\rightarrow G}$–/– embryos. We used in situ hybridization to ask if R/C identity was forming in the absence of presenilin. We find that caudal identity (*Uncx4.1*) was not restored in *PS1–/–; PS2–/–;*$N1^{V\rightarrow G}$–/– embryos (Figure 3J). In this regard, they were identical to *PS1–/–;PS2–/–* embryos (Figure 3I; Donoviel et al., 1999). Since all the triple homozygote embryos were developmentally retarded (as expected from *presenilin*-deficient animals), we verified that segmentation occurred by SEM on both fresh-fixed embryos and embryos analyzed after treatment for in situ hybridization (see Experimental Procedures). *PS1–/–; PS2–/–* embryos may not form somites (Donoviel et al., 1999), but did form periodic condensation (Figures 4E and 4F; Herreman et al., 1999), indicating that some clock activity remained (perhaps due to Wnt3A, see Discussion; Aulehla and Herrmann, 2004). This observation was used to argue that Notch proteolysis was a critical component of the segmentation clock. The observation that *PS1–/–;PS2–/–;*$N1^{V\rightarrow G}$–/– triple mutant embryos are able to form a few normal-looking anterior somites in the absence of γ-secretase (Figures 4H and 4H′) might suggest that a processing-independent signaling function of Notch existed. However, such an activity of Notch (i.e., residual segmentation in *PS1–/–; PS2–/–* mice; Figures 4E and 4F) was not observed despite the presence of all Notch proteins. The fact that segmentation in *PS1–/–;PS2–/–* embryos is only observed in the presence of $N1^{V\rightarrow G}$ could only be possible if this conservative substitution of an amino acid within the transmembrane domain had endowed $N1^{V\rightarrow G}$ with a neomorphic activity. Since in most assays, $N1^{V\rightarrow G}$ suppresses rather than enhances signaling from other Notch molecules (see Supplemental Figures S1 and S2), the most likely activity provided by $N1^{V\rightarrow G}$ in this context would be the low-level production of NICD via an alternative protease.

The Activity Provided by $N1^{V\rightarrow G}$ Is Mediated by an Unknown Protease

Previous studies were able to detect Notch activation in PS1 and PS2 double-deficient cells (PSDKO) using reporter assays (Berechid et al., 2002). Evidence that cells contain a Notch-cleaving activity separable from presenilin has also been reported (Taniguchi et al., 2002). Three families of I-CLiPs exist that have the ability to cleave intramembranous proteins (Golde and Eckman, 2003; Martoglio and Golde, 2003; Weihofen and Martoglio, 2003); any of these or an unknown I-CLiP could recognize $N1^{V\rightarrow G}$ (but not N1) as a sub-

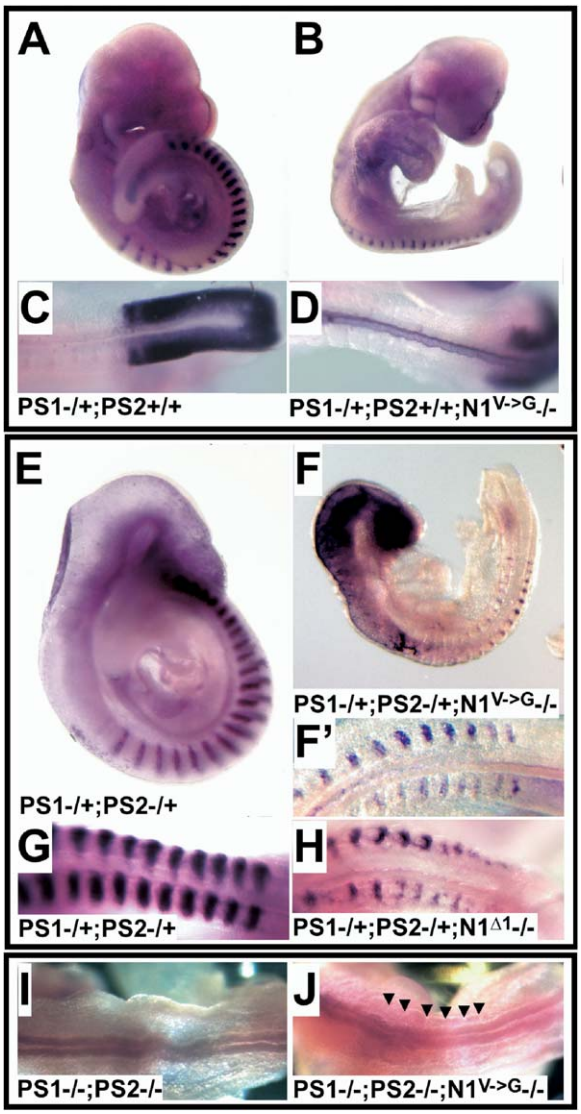

Figure 3. *presenilin* Dosage Modifies the Phenotype of $N1^{V\rightarrow G}$–/– but Not $N1^{\Delta 1}$–/–

(A, B, E–J) Whole-mount in situ hybridization for *Uncx4.1* of ~E9.5 embryos, genotypes labeled within panels.

(C and D) Whole-mount in situ hybridization for Delta1 of ~E9.5 embryos, genotypes labeled within panels. Littermates are shown at the same magnification: (A) and (B), (C) and (D), (E) and (F), (G) and (H), and (I) and (J). Posterior is to the right (C, D, and F′–J).

strate by virtue of the helix-relaxing Gly inserted in its TMD (Lemberg and Martoglio, 2002; Urban and Freeman, 2003). To examine the dependence of $N1^{V\rightarrow G}$ proteolysis on γ-secretase, we performed tissue culture experiments with wild-type and genetically defined PSDKO mouse embryonic fibroblasts (MEFs) (Figure 5A; Herreman et al., 2003). To maximize NICD retrieval (Schroeter et al., 1998), wild-type MEFs were transiently transfected with Flag-RBP (Waltzer et al., 1995) and constitutively active, membrane-tethered forms of Notch (N1ΔE6mt or $N1\Delta E^{V\rightarrow G}$6mt). To block Notch proteolysis, we chose the γ-secretase inhibitor DAPT (Dovey et al., 2001) because it is specific for presenilin and does not inhibit the activity of SPP (a related aspartyl protease; Weihofen et al., 2003). As reported, DAPT blocked most of the NICD formation in wild-type MEFs (Figure 5A). Coimmunoprecipitation of NICD generated from $N1\Delta E^{V\rightarrow G}$ with Flag-RBP followed by anti-Myc immunoblotting demonstrated that residual proteolysis of $N1^{V\rightarrow G}$ was γ-secretase dependent and inhibited by DAPT; that conclusion was further supported by lack of NICD recovery from PSDKO MEFs (Figure 5A; Armogida et al., 2001; Berechid et al., 2002; Herreman et al., 2000; Saxena et al., 2001; Zhang et al., 2000). These results also indirectly demonstrate that SPP is not able to efficiently cleave the $N1\Delta E^{V\rightarrow G}$ substrate. We considered the possibility that PSH proteins (Ponting et al., 2002) are able to cleave $N1^{V\rightarrow G}$. PSH1, 3 (=SPP; Weihofen et al., 2002), 4, and 5 were unable to alter N1ΔE or $N1\Delta E^{V\rightarrow G}$ proteolysis in PSDKO MEFs (data not shown). This result is not surprising considering that PSH proteins have an inverted topology relative to presenilin proteins, and their substrates are Type II membrane proteins (Nyborg et al., 2004).

To determine if any unknown activity was able to preferentially recognize $N1\Delta E^{V\rightarrow G}$ as a substrate, we examined the ability of $N1\Delta E^{V\rightarrow G}$ and N1ΔE to activate the TP1::Luciferase (TP1::Luc; Minoguchi et al., 1997) reporter, chosen because it is the most sensitive reporter for NICD activity that we have (Supplemental Figure S4). Interestingly, $N1\Delta E^{V\rightarrow G}$ was able to activate TP1::Luc 56%–100% better than N1ΔE in independent experiments (Figure 5B). This would be consistent with an activity preferentially utilizing $N1\Delta E^{V\rightarrow G}$ as a substrate in PSDKO MEFs. The difference in TP1::Luc activation is not due to differences in protein expression level (data not shown). We hypothesized that NICD produced from $N1^{V\rightarrow G}$ by the unknown protease is less abundant than that produced by γ-secretase, reaching activation thresholds only in the PSM. To test this hypothesis, we cotransfected PS1 and TP1::luc with $N1\Delta E^{V\rightarrow G}$ in PSDKO MEFs. Indeed, higher luciferase levels were observed, confirming that while $N1\Delta E^{V\rightarrow G}$ is a poor γ-secretase substrate, the unknown protease remains much less efficient than γ-secretase, even in the context of the V→G mutation (Figure 5C).

Our data indicate that the absence of presenilin protein and the presence of a V1744G mutation in Notch allowed a protease to assume the role of γ-secretase in Notch proteolysis. Others have also reported production of Aβ (Wilson et al., 2003) and activation of Hes1-luciferase (Berechid et al., 2002) by a membrane-tethered Notch (ΔE) in PSDKO cells. When proteases are inhibited or missing, other proteases may emerge as capable of providing functional compensation. One example includes mammalian cells adapted for growth in the presence of proteasome inhibitors; these cells activate other proteases to functionally replace the proteasome (Geier et al., 1999; Glas et al., 1998). We do not know the identity of the "unknown protease," but a candidate exists. Crouthamel et al., (2002) have characterized a novel aspartyl-protease, γ-three protease, capable of cleaving APP to produce Aβ peptide and release NICD from N1ΔE. At this time γ-three has not been cloned; importantly, γ-three is not a factor in Notch signaling since NICD is not released in sufficient

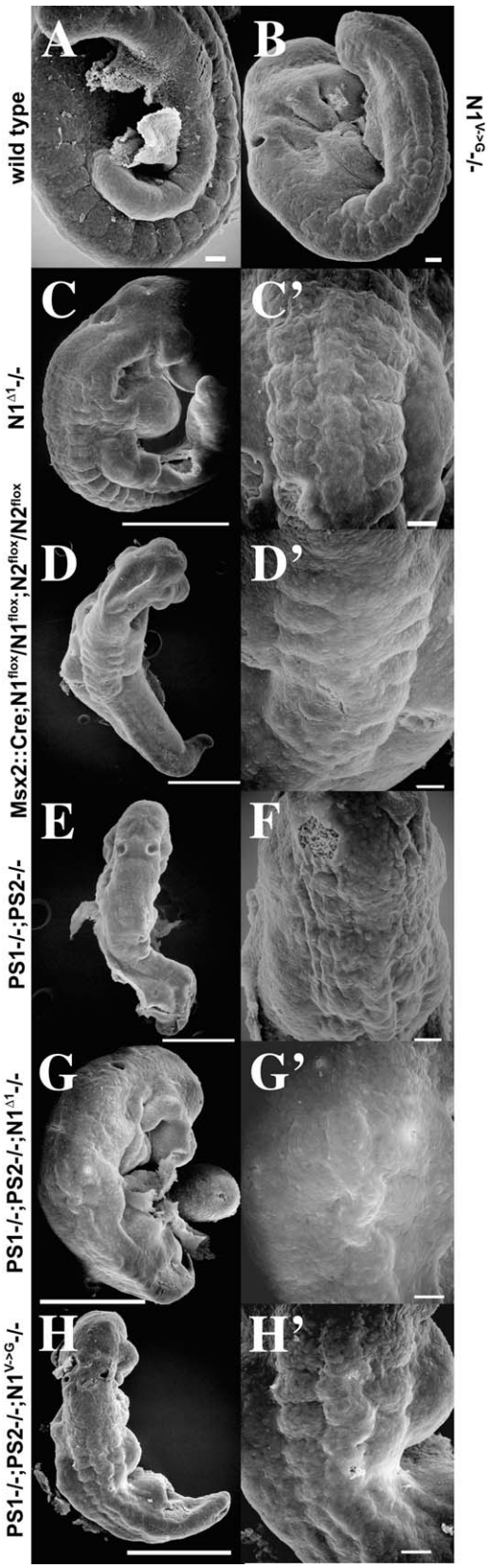

Figure 4. Comparing Phenotypes of Notch and Presenilin Allele Combinations

SEM images of ~E9.5 embryos. Magnification bars shown in images (A), (B), (C′), (D′), (F), (G′), and (H′) 50 units, and in (C), (D), (E), (G), and (H) 500 units. Higher magnification images (C′, D′, F, G′, and H′) are at the level of the forelimb bud. Genotypes are as follows: (A) wild-type littermate of (B) $N1^{V\rightarrow G}$–/–, (C and C′) $N1^{\Delta 1}$–/– same embryo, (D and D′) *Msx2::Cre;*$N1^{flox}/N1^{flox}$;$N2^{flox}/N2^{flox}$ same embryo, (E and F) *PS1–/–;PS2–/–* different embryos, (G and G′) *PS1–/–;PS2–/–;*$N1^{\Delta 1}$–/– same embryo, and (H and H′) *PS1–/–;PS2–/–;*$N1^{V\rightarrow G}$–/– same embryo.

amounts from native Notch1 or Notch2 in *PS1–/–;PS2–/–* PSM.

Presenilin Has a Function during Segmentation Independent of Notch and γ-Secretase

If the role of PS1 and PS2 in somitogenesis was solely to act as a protease in the Notch pathway, we reasoned that the phenotype resulting from removing all Notch activity should resemble that of *PS1–/–;PS2–/–* embryos. Since only *Notch1* and *Notch2* genes are expressed in the PSM, we generated *Notch1* and *Notch2* double null embryos using conditional alleles of both genes (Pan et al., 2004; Yang et al., 2004) and maternal deletion (in ovo) using the *Msx2::Cre* transgene, which has leaky expression in the oocyte (Y. Pan and R.K., unpublished observation; see also Hayashi et al., 2003; Vincent and Robertson, 2003), to determine if germline deletion of Notch genes normally expressed in the PSM phenocopies complete loss of presenilin. Surprisingly, *Msx2::Cre;*$N1^{flox}/N1^{flox}$;$N2^{flox}/N2^{flox}$ embryos (henceforth N1N2DKO) differ from the unsegmented *PS1–/–;PS2–/–* embryos (Figures 4D–4F): they instead resemble the zebrafish *notch1a* (*deadly seven*; Holley et al., 2002) and *deltaD* (*after eight*; Holley et al., 2000) mutants. The N1N2DKO embryos produce 7–9 somites (to the region of the forelimb bud), reminiscent of $Wnt3A^{neo}$–/– embryos (Greco et al., 1996; Takada et al., 1994), and an unsegmented extended posterior region (Figure 4D). Lack of anterior somitogenesis in *PS1–/–;PS2–/–* embryos but its presence in N1N2DKO could indicate a requirement for a presenilin function that is unrelated to Notch proteolysis; alternatively, in the absence of *Notch1* and *Notch2*, another Notch protein (Notch 3 or 4), expressed below the detection ability of in situ hybridization, may produce sufficient NICD to rescue anterior somites.

To address this issue, embryos deficient in other γ-secretase components, *Nicastrin* (*NCT*), *Pen-2*, and *APH-1a* need to be examined. Together with presenilin, these three proteins play an essential role in γ-secretase, and in their absence, Notch signaling is not detected (Francis et al., 2002; Li et al., 2003a). *NCT*-deficient embryos were described as having abnormal somites (Li et al., 2003a; Li et al., 2003b). Stereoscopic images of *NCT–/–* embryos clearly differ from *PS1–/–;PS2–/–* in having visible clefts (4–6 somites), *Pen-2* null embryos have 5–8 anterior somites (C.-A. Mao and J. Nye, personal communication), and *APH-1a* mutants have 15–17 somites (Ma et al., 2005; Serneels et al., 2005). Since embryos deficient in γ-secretase due to mutations in every other essential component of this enzyme form somite clefts, we believe this indicates

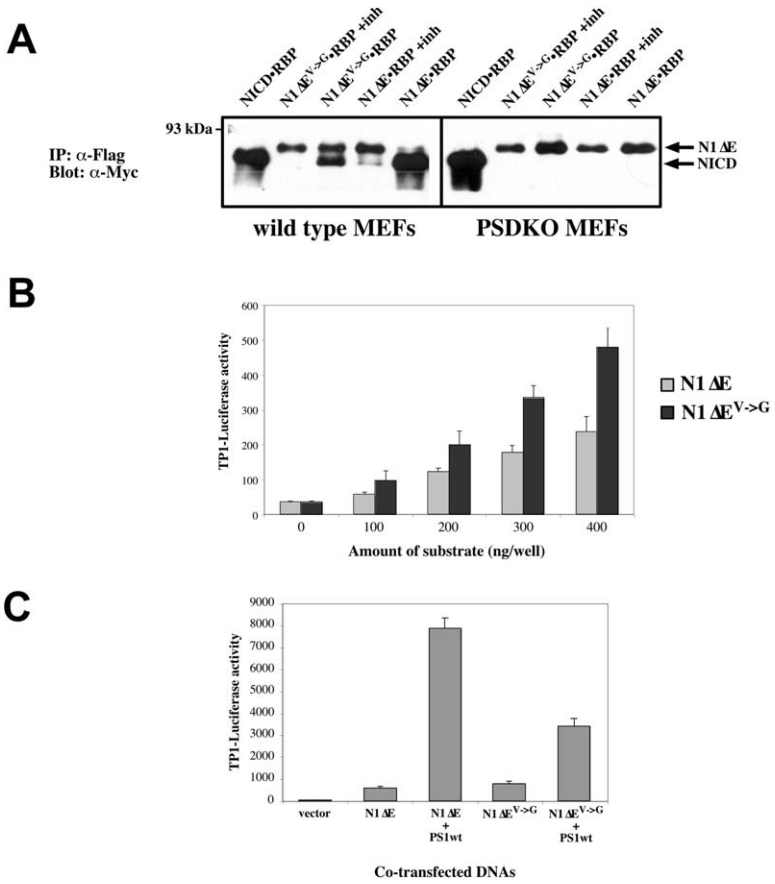

Figure 5. Analyses of the Proteolysis of $N1^{V\rightarrow G}$ in Presenilin-Deficient Cells

(A) Residual proteolysis of $N1^{V\rightarrow G}$ is presenilin/γ-secretase dependent: wild-type and PSDKO MEFs were transfected with Flag-RBP and constitutively active, membrane-tethered forms of Notch (N1ΔE6mt or $N1\Delta E^{V\rightarrow G}$6mt) and then treated with the γ-secretase inhibitor DAPT or DMSO control. Cell lysates were coimmunoprecipitated with anti-Flag antibodies and NICD production was detected by anti-Myc.

(B) $N1\Delta E^{V\rightarrow G}$ can activate the TP1::Luc reporter better than N1ΔE in PSDKO cells. Shown is a representative experiment performed in quadruplicate (n = 5).

(C) TP1::Luc activation by N1ΔE and $N1\Delta E^{V\rightarrow G}$ when coexpressed with wild-type PS1. Even though $N1\Delta E^{V\rightarrow G}$ is a poor substrate for presenilin/γ-secretase, presenilin/γ-secretase is more efficient than the "unknown protease." Shown is a representative experiment (n = 5) done in triplicate.

Error bars represent standard deviation.

that presenilin is required to perform a task unrelated to γ-secretase.

Discussion

Segmentation in the Mouse Shows the Predicted Low Sensitivity to the Level of NICD

Evidence that the threshold for NICD in the PSM is lower than that required in other tissue is provided in the demonstration that $N1^{V\rightarrow G}$ acts as a modifier of *Delta1* (see Figure 2 and Supplemental Figure S2), reducing Notch signaling and enhancing the *Notch1* phenotype in all tissues—except for anterior and trunk somites. Interestingly, sensitivity to NICD levels increases in a posterior progression: trunk somites require more NICD than anterior somites (anterior somites form in *N1N2DKO* null embryos; Figure 4) and tail bud somitogenesis requires more NICD than trunk somitogenesis ($N1^{V\rightarrow G}$ enhances a kinky tail phenotype in a heterozygous *Dll1* background; Supplemental Figure S2). We also observed only mild effects in somites of $N1^{V\rightarrow G}$*–/–* embryos with various combinations of *presenilin* alleles that have increased severity of the neural phenotype (Figure 3). These observations would be consistent with an increased need for NICD-mediated synchronization of a free oscillating cellular clock for later-born cells.

The phenotype of *PS1–/–;PS2–/–;*$N1^{V\rightarrow G}$*–/–* embryos does not improve relative to that of *PS1–/–; PS2–/–* embryos in any respect other than segmentation (Figure 4). Therefore, these results suggest that the PSM has the *lowest* functional threshold for NICD compared to other tissues. Furthermore, combined with the fact that *NCT, Pen-2*, and *APH-1a* embryos have anterior somites, these data propose that presenilin has roles *independent* from γ-secretase and that the unknown protease (Figure 5) does not provide enough NICD to reach the threshold of activation in other tissues.

NICD Contribution in the PSM

The models predicted that the canonical Notch pathway contributes to the segmentation clock, perhaps even constituting the clock mechanism (Dale et al., 2003; Maroto and Pourquie, 2001), as well as contributing to somite clefting (Sato et al., 2002) and to acquisition of R/C polarity (Saga and Takeda, 2001; Takahashi et al., 2003). We provide visual evidence of cycling NICD production in the PSM (Figure 1), consistent with some prediction of these models and the observations that mRNA and protein of Notch target genes cycle (*Lfng, Hes1, Hes5,* and *Hes7*; Bessho et al., 2003; Dale et al., 2003; Hirata et al., 2004).

While we are able to demonstrate that NICD cycles in the middle of the PSM and resolves to a stripe in the region straddling the future boundary of S-1 and S0, we could not detect NICD localization in all the areas where it is predicted to function. We were not able to detect a consistent NICD signal in the very posterior of

the PSM where cycling of target genes is initiated. Two opposing hypotheses could explain the lack of NICD in the posterior region. First, the amounts of NICD in the most posterior region may be too low for the Val1744 antibody to detect. Alternatively, NICD may not be required in the posterior region to initiate the cycling.

The initiation of the segmentation clock could be under control of Wnt3a (Aulehla and Herrmann, 2004; Aulehla et al., 2003), Hes1 (Hirata et al., 2002), Hes7 (Hirata et al., 2004), a Notch-independent contribution of presenilin, or a yet unidentified player. *Notch2* could not be responsible for the target gene initiation: although it is expressed in the PSM, its expression domain does not extend to the posterior of the PSM, nor are there any segmentation defects visible in the null embryo (Hamada et al., 1999). Moreover, *N1N2DKO* embryos form 7 to 9 somites (Figure 4), and somites form in *RBP-Jκ* null embryos (Oka et al., 1995). While our data do not support a model in which Notch signaling initiates the cycle, our observations support the model based on the zebrafish embryos that argue that Notch activation is clearly required to reinforce/refine the pattern after the first few somites have formed (Jiang et al., 2000; Lewis, 2003).

Presenilin Has Roles in Somitogenesis Independent of Notch and γ-Secretase

The phenotypic consequence of Notch pathway loss in the process of somitogenesis resembles that of *NCT*, *Pen-2*, and *APH-1a* mutants (components of γ-secretase): all form at least 4 to 9 anterior somites. In contrast, loss of both PS1 and PS2 (the catalytic component of γ-secretase) results in lack of anterior segmentation but formation of periodic condensations. This observation suggests that presenilin may have other roles in somitogenesis independent of γ-secretase and Notch. Previous data have demonstrated the formation of clefts in the absence of *Notch1* and *Notch3* (Krebs et al., 2003), and we show in this manuscript that clefts form in the absence of *Notch1* and *Notch2* (N1N2DKO; Figure 4). Therefore, clefts are still able to form in the absence of Notch pathway receptors and the common nuclear cofactor, *RBP-Jκ* (abolishing canonical signaling from all four Notch paralogs) (Oka et al., 1995). The severity of the phenotype caused by the loss of both PS1 and PS2 is consistent with loss of γ-secretase activity in addition to an activity different from its role as a protease. It is remotely possible that the ability of null mutants of γ-secretase components (*NCT*, *Pen-2*, and *APH-1a*) to form a few anterior somites could be due to a residual γ-secretase activity of the presenilin, the catalytic component of γ-secretase. However, two lines of evidence argue against that possibility. First, reconstitution of γ-secretase activity requires all four components (NCT, Pen-2, APH-1a, and presenilin) (Edbauer et al., 2003; Takasugi et al., 2003). Second, even the slightest amount of NICD in *PS1−/−;PS2−/−;N1*$^{V\rightarrow G}$*−/−* embryos (Figure 4) can rescue anterior somites. If Notch (a better presenilin substrate than N$^{V\rightarrow G}$; Figure 5) could be cleaved at all by the remaining components, the extreme sensitivity of the PSM to NICD would have allowed anterior somites to form. Thus, we conclude that no NICD is produced in γ-secretase-deficient, presenilin-containing PSM.

Collectively, this report as well as published observations by others is consistent with two different models for the γ-secretase-independent function of presenilin in somitogenesis. The first hypothesizes that three pathways (NICD, Wnt, and presenilin) play redundant roles during anterior somitogenesis. The second (which we favor) hypothesizes that presenilin couples NICD- and Wnt-based clocks.

The first model proposes that NICD (normally produced by γ-secretase), Wnt signaling, and the presenilin activity play redundant roles in anterior somitogenesis but are all required in trunk and tail somites. This could reflect progressive loss in synchronicity in individual, oscillating, later-born PSM cells. The lack of anterior somitogenesis in *PS1−/−;PS2−/−* embryos (which lack NICD1/2/3/4 and presenilin but have Wnt3a) but the presence of anterior somitogenesis in N1N2DKO, *Nicastrin, APH-1a,* and *Pen-2* mutants (which lack NICD1/2/3/4 but have Wnt3a and presenilin) indicates a requirement for a presenilin function that is unrelated to Notch proteolysis or to γ-secretase. Intriguingly, any two of these three activities are sufficient to synchronize the oscillator: restoring Notch proteolysis in *PS1−/−;PS2−/−;N1*$^{V\rightarrow G}$*−/−* embryos (which now have NICD1 and Wnt3a but no presenilin) restores anterior somitogenesis, indicating that in the presence of Wnt and NICD, the role of presenilin is redundant in the anterior. One might imagine a hypothetical genotype lacking periodic condensations in the anterior. Such a hypothetical mutant would lack clock initiation; perhaps a triple Wnt3a/PS1/PS2 animal will have this phenotype.

The second model proposes that presenilin provides a coupling between NICD and Wnt signaling by contributing to both clocks. Presenilin proteins have been linked to functions regulating protein trafficking (Wrigley et al., 2004), PI3K activity (Baki et al., 2004), and β-catenin degradation (Kang et al., 2002). The latter functions may be the result of altered trafficking. FAD mutations such as M146L and ΔE9 perform the γ-secretase function of presenilin but not β-catenin degradation (Soriano et al., 2001) or AKT phosphorylation (Baki et al., 2004). It remains to be seen if they impact the trafficking functions of presenilin. The observation that replacing PS1 with PS1$^{M146L\text{-}Neo}$ results in highly penetrant segmentation defects with no significant impact on other tissues (Rozmahel et al., 2002a; Rozmahel et al., 2002b) suggests that PS2 and PS1^{M146L} provide function inefficiently during the process of somitogenesis, despite PS1^{M146L}'s ability to execute Notch cleavage as well as PS1 (Schroeter et al., 2003).

Therefore, we propose that presenilin would contribute to Notch activation as γ-secretase via cleavage, and to the Wnt clock by its trafficking function that in turn impacts β-catenin stability, regulation of GSK3β activity by AKT, or both. In the absence of presenilin, Notch cleavage is lost and β-catenin cycling is compromised due to either loss of a constitutive degradation scaffold or degraded due to enhanced GSK3β activity. The lack of anterior somitogenesis in *PS1−/−;PS2−/−* embryos is thus due to a loss of cycling from both NICD and Wnt clocks.

Experimental Procedures

Mouse Lines

PS1, *PS2* (Herreman et al., 1999), *Dll1*^{lacZ} (de Angelis et al., 1997), *N1*$^{\Delta 1}$ (Conlon et al., 1995), and *N1*$^{V\rightarrow G}$ (Huppert et al., 2000) were

maintained as heterozygotes in a CD-1 background (Charles River Laboratories, Wilmington, MA). *RBP-J$_K$* (Oka et al., 1995) was maintained as heterozygotes in a C57BL/6J background. *Msx2::cre* (Sun et al., 2000), *N1^{flox}* (made in R. Kopan lab) (Pan et al., 2004; Yang et al., 2004), and *N2^{flox}* (made in the T. Gridley lab) (Pan et al., 2004) are mixed backgrounds of C57BL/6J and 129SvJ.

Analysis of Mice

Heterozygous *N1$^{V\rightarrow G}$* animals were crossed with either heterozygous *RBP-J$_K$* or *Dll1^{lacZ}* animals. Litters were photographed, weights were taken at postnatal day 7, and tail DNA was used to genotype. Primers for genotyping can be found in the references listed with each mouse line above.

Analysis of Embryos: In Situ Hybridization, PSM Cultures, and SEM

Embryos were extracted at E9 to E10 and yolk sac DNA was used for genotyping. Primers for genotyping can be found in the references listed with each mouse line above.

In situ hybridization was performed as in Huppert et al. (2000). Probes used for hybridization were *Uncx4.1* (Neidhardt et al., 1997), *Dll1* (provided by D. Henrique), and *Hes7* (provided by R. Kageyama).

PSM cultures were performed as in Correia and Conlon (2000). Briefly, E10.5 PSM were removed from the embryo and bisected. One half was fixed immediately in Bouin's fix, and the other half was incubated for 60 min and then Bouin's fixed.

For SEM analysis, mouse embryos were fixed with 4% paraformalydehyde fresh or postfixed after in situ hybridization with 4% formaldehyde + 0.1% glutaraldehyde. Embryos were then postfixed with 1% aqueous osmium tetroxide for 2.5 hr, dehydrated in an ethyl alcohol series, and critical point dried in liquid CO_2. Mounted samples were sputter-coated with 30 nm of gold and examined in a Hitachi S-450 scanning electron microscope operated at 20 kV accelerating voltage.

Val1744 Antibody: Immunofluorescence and Western

Immunofluorescence was performed on paraffin sections of E10.5 PSM as in Cheng et al. (2003). Tissue was Bouin's fixed and treated for paraffin sectioning, and the primary antibody Val1744 (Cell Signaling Technology, a division of New England Biolabs) was used at 1:300.

Transfections of HEK293T cells and Western analysis performed as in Schroeter et al. (1998). The ΔE Notch homolog constructs are described in Saxena et al. (2001). Western blot incubated with the primary Val1744 (Cell Signaling Technology, a division of New England Biolabs) at 1:1000 in 5% milk overnight at 4°C. The secondary anti-Rabbit-HRP (Amersham Pharmacia Biotech) used at 1:5000 in 0.1% Tween-20. The same blot was then stripped, reblocked, and incubated with the primary antibody 9E10 (1:1000 in 5% milk; Myc hybridoma) followed by secondary anti-Mouse-HRP (1:5000 in 0.1% Tween-20; Amersham Pharmacia Biotech).

Cell Culture

Wild-type and PSDKO MEFs were grown and maintained in DMEM supplemented with 10% FBS. MEFs were transfected using FuGene 6 (Roche) or Lipofectamine 2000 (Invitrogen) following the manufacturer's protocols. Coimmunoprecipitations performed as in Schroeter et al. (2003).

For reporter assay experiments, PSDKO cells were seeded in 24-well plates at a density of 4.25×10^4 cells/well. After 24 hr, each well was transfected with 100 ng luciferase reporter (4xCSL, Hes1, or TP1) and 50 ng CS2+βgal, along with 200 ng empty vector or Notch expression construct (NICD, N1ΔE, or N1ΔE$^{V\rightarrow G}$) and CS2+ plasmid as carrier DNA (up to 1000 ng/well). 24 hr after transfection, luciferase and β-galactosidase assays were performed as described (Saxena et al., 2001). All experiments were done in quadruplicate. Luciferase activity was normalized against β-galactosidase activity to correct for differences due to transfection efficiency.

Supplemental Data

Supplemental Data include four figures and can be found with this article online at http://www.developmentalcell.com/cgi/content/full/8/5/677/DC1/.

Acknowledgments

We wish to thank R. Conlon, T. Gridley, A. Gossler, T. Honjo, and G. Martin for providing animals; L. Strobl, U. Zimber-Strobl, T. Honjo, D. Henrique, B. Herrmann, R. Kageyama, A. Nyborg, and T. Golde for providing reagents; A. Gossler, C.-A. Mao, and J.S. Nye for sharing unpublished data; M. Veith for SEM analysis (Washington University Department of Biology Service Laboratory); B. Coleman, A. Johnson, and M. Scott for paraffin sections (Washington University Department of Pharmacology Histology Core); J.K. Dale and O. Pourquie Lab (Stowers Institute) for help with in situ hybridization; A. Nichols, D. Pooran, G. Rice, and T. Shen for help with genotyping; Y. Pan, L. Steire, and Y. Wu for help with mice; O. Ilagan for statistical analysis; C. Smith for rederivation of mouse lines; and O. Pourquie for reading and commenting on manuscript. S.S.H. and R.K. were supported by NIH GM55479-09 and HD044056-01, R.K. and M.X.G.I. were supported by Alzheimer's Association IIRG-03-5283, and B.D.S. was supported by the Alzheimer's Association (Pioneer Award) and K.U. Leuven (GOA).

Received: October 11, 2004
Revised: December 23, 2004
Accepted: February 17, 2005
Published: May 2, 2005

References

Aoyama, H., and Asamoto, K. (2000). The developmental fate of the rostral/caudal half of a somite for vertebra and rib formation: experimental confirmation of the resegmentation theory using chick-quail chimeras. Mech. Dev. *99*, 71–82.

Arias, A.M., Zecchini, V., and Brennan, K. (2002). CSL-independent Notch signalling: a check point in cell fate decisions during development? Curr. Opin. Genet. Dev. *12*, 524–533.

Armogida, M., Petit, A., Vincent, B., Scarzello, S., da Costa, C.A., and Checler, F. (2001). Endogenous β-amyloid production in presenilin-deficient embryonic mouse fibroblasts. Nat. Cell Biol. *3*, 1030–1033.

Aulehla, A., and Herrmann, B.G. (2004). Segmentation in vertebrates: clock and gradient finally joined. Genes Dev. *18*, 2060–2067.

Aulehla, A., and Johnson, R.L. (1999). Dynamic expression of *lunatic fringe* suggests a link between Notch signaling and an autonomous cellular oscillator driving somite segmentation. Dev. Biol. *207*, 49–61.

Aulehla, A., Wehrle, C., Brand-Saberi, B., Kemler, R., Gossler, A., Kanzler, B., and Herrmann, B.G. (2003). *Wnt3a* plays a major role in the segmentation clock controlling somitogenesis. Dev. Cell *4*, 395–406.

Baki, L., Shioi, J., Wen, P., Shao, Z., Schwarzman, A., Gama-Sosa, M., Neve, R., and Robakis, N.K. (2004). PS1 activates PI3K thus inhibiting GSK-3 activity and tau overphosphorylation: effects of FAD mutations. EMBO J. *23*, 2586–2596.

Berechid, B.E., Kitzmann, M., Foltz, D.R., Roach, A.H., Seiffert, D., Thompson, L.A., Olson, R.E., Bernstein, A., Donoviel, D.B., and Nye, J.S. (2002). Identification and characterization of presenilin-independent Notch signaling. J. Biol. Chem. *277*, 8154–8165.

Bessho, Y., and Kageyama, R. (2003). Oscillations, clocks and segmentation. Curr. Opin. Genet. Dev. *13*, 379–384.

Bessho, Y., Sakata, R., Komatsu, S., Shiota, K., Yamada, S., and Kageyama, R. (2001). Dynamic expression and essential functions of Hes7 in somite segmentation. Genes Dev. *15*, 2642–2647.

Bessho, Y., Hirata, H., Masamizu, Y., and Kageyama, R. (2003). Periodic repression by the bHLH factor Hes7 is an essential mechanism for the somite segmentation clock. Genes Dev. *17*, 1451–1456.

Bettenhausen, B., de Angelis, M.H., Simon, D., Guenet, J.-L., and Gossler, A. (1995). Transient and restricted expression during mouse embryogenesis of *Dll1*, a murine gene closely realted to *Drosophila Delta*. Development *121*, 2407–2418.

Brennan, K., and Gardner, P. (2002). Notching up another pathway. Bioessays *24*, 405–410.

Bussen, M., Petry, M., Schuster-Gossler, K., Leitges, M., Gossler,

A., and Kispert, A. (2004). The T-box transcription factor Tbx18 maintains the separation of anterior and posterior somite compartments. Genes Dev. *18*, 1209–1221.

Cheng, H.-T., Miner, J.H., Lin, M., Tansey, M.G., Roth, K., and Kopan, R. (2003). γ-Secretase activity is dispensable for mesenchyme-to-epithelium transition but required for podocyte and proximal tubule formation in developing mouse kidney. Development *130*, 5031–5042.

Conlon, R.A., Reaume, A.G., and Rossant, J. (1995). *Notch1* is required for the coordinate segmentation of somites. Development *121*, 1533–1545.

Cordes, R., Schuster-Gossler, K., Serth, K., and Gossler, A. (2004). Specification of vertebral identity is coupled to Notch signalling and the segmentation clock. Development *131*, 1221–1233.

Correia, K.M., and Conlon, R.A. (2000). Surface ectoderm is necessary for the morphogenesis of somites. Mech. Dev. *91*, 10–30.

Crouthamel, M.-C., Gardell, S.J., Huang, Q., Lai, M.-T., and Li, Y. (2002). Gamma Three Protease http://www.wipo.int/cgi-pct/guest/ifetch5?ENG+PCT-ALL.vdb+14+1104408-SCORE+256+2+25312+BASICHTML-ENG+29+25275+26+25+SEP-0/HITNUM,B,,SCORE+gamma+OR+three+OR+protease.

Dale, J.K., Maroto, M., Dequeant, M.-L., Malapert, P., McGrew, M., and Pourquie, O. (2003). Periodic Notch inhibition by Lunatic Fringe underlies the chick segmentation clock. Nature *421*, 275–278.

de Angelis, M.H., McIntyre, J.I., and Gossler, A. (1997). Maintenance of somite borders in mice requires the Delta homologue Dll1. Nature *386*, 717–721.

de la Pompa, J.L., Wakeham, A., Correia, K.M., Samper, E., Brown, S., Aguilera, R.J., Nakano, T., Honjo, T., Mak, T.W., Rossant, J., and Conlon, R.A. (1997). Conservation of the Notch signalling pathway in mammalian neurogenesis. Development *124*, 1139–1148.

Donoviel, D.B., Hadjantonakis, A.-K., Ikeda, M., Zheng, H., Hyslop, P.S.G., and Bernstein, A. (1999). Mice lacking both presenilin genes exhibit early embryonic patterning defects. Genes Dev. *13*, 2801–2810.

Dovey, H.F., John, V., Anderson, J.P., Chen, L.Z., de Saint Andrieu, P., Fang, L.Y., Freedman, S.B., Folmer, B., Goldbach, E., Holsztynska, E.J., et al. (2001). Functional gamma-secretase inhibitors reduce beta-amyloid peptide levels in brain. J. Neurochem. *76*, 173–181.

Edbauer, D., Winkler, E., Regula, J.T., Pesold, B., Steiner, H., and Haass, C. (2003). Reconstitution of gamma-secretase activity. Nat. Cell Biol. *5*, 486–488.

Forsberg, H., Crozet, F., and Brown, N.A. (1998). Waves of mouse *Lunatic fringe* expression, in four-hour cycles at two-hour intervals, precede somite boundary formation. Curr. Biol. *8*, 1027–1030.

Francis, R., McGrath, G., Ahang, J., Ruddy, D.A., Sym, M., Apfeld, J., Nicoll, M., Maxwell, M., Hai, B., Ellis, M.C., et al. (2002). *aph-1* and *pen-2* are required for Notch pathway signaling, γ-secretase cleavage of ßAPP, and presenilin protein accumulation. Dev. Cell *3*, 85–97.

Galceran, J., Sustmann, C., Hsu, S.-C., Folberth, S., and Grosschedl, R. (2004). LEF1-mediated regulation of Delta-like1 links Wnt and Notch signaling in somitogenesis. Genes Dev. *18*, 2718–2723.

Geier, E., Pfeifer, G., Wilm, M., Lucchiari-Hartz, M., Baumeister, W., Eichmann, K., and Niedermann, G. (1999). A giant protease with potential to substitute for some functions of the proteasome. Science *284*, 978–981.

Giudicelli, F., and Lewis, J. (2004). The vertebrate segmentation clock. Curr. Opin. Genet. Dev. *14*, 407–414.

Glas, R., Bogyo, M., McMaster, J.S., Gaczynska, M., and Ploegh, H.L. (1998). A proteolytic system that compensates for loss of proteasome function. Nature *392*, 618–622.

Golde, T.E., and Eckman, C.B. (2003). Physiologic and pathologic events mediated by intramembranous and juxtamembranous proteolysis. Sci. STKE *172*, RE4.

Greco, T.L., Takada, S., Newhouse, M.M., McMahon, J.A., McMahon, A.P., and Camper, S.A. (1996). Analysis of the vestigial tail mutation demonstrates that Wnt-3a gene dosage regulates mouse axial development. Genes Dev. *10*, 313–324.

Hamada, Y., Kadokawa, Y., Okabe, M., Ikawa, M., Coleman, J.R., and Tsujimoto, Y. (1999). Mutation in ankyrin repeats of the mouse Notch2 gene induces early embryonic lethality. Development *126*, 3415–3424.

Hayashi, S., Tenzen, Y., and McMahon, A.P. (2003). Maternal inheritance of Cre activity in a Sox2Cre deleter strain. Genesis *37*, 51–53.

Herreman, A., Hartmann, D., Annaert, W., Saftig, P., Craessaerts, K., Serneels, L., Umans, L., Schrijvers, V., Checler, F., Vanderstichele, H., et al. (1999). Presenilin 2 deficiency causes a mild pulmonary phenotype and no changes in amyloid precursor protein processing but enhances the embryonic lethal phenotype of presenilin 1 deficiency. Proc. Natl. Acad. Sci. USA *96*, 11872–11877.

Herreman, A., Serneels, L., Annaert, W., Collen, D., Schoonjans, L., and Strooper, B.D. (2000). Total inactivation of γ-secretase activity in presenilin-deficient embryonic stem cells. Nat. Cell Biol. *2*, 461–462.

Herreman, A., Gassen, G.V., Bentahir, M., Nyabi, O., Craessaerts, K., Mueller, U., Annaert, W., and Strooper, B.D. (2003). γ-Secretase activity requires the presenilin-dependent trafficking of nicastrin through the Golgi apparatus but not its complex glycosylation. J. Cell Sci. *116*, 1127–1136.

Hirata, H., Bessho, Y., Kokubu, H., Masamizu, Y., Yamada, S., Lewis, J., and Kageyama, R. (2004). Instability of Hes7 protein is crucial for the somite segmentation clock. Nat. Genet. *36*, 750–754.

Hirata, H., Yoshiura, S., Ohtsuka, T., Bessho, Y., Harada, T., Yoshikawa, K., and Kageyama, R. (2002). Oscillatory expression of the bHLH factor Hes1 regulated by a negative feedback loop. Science *298*, 840–843.

Hofmann, M., Schuster-Gossler, K., Watabe-Rudolph, M., Aulehla, A., Herrmann, B.G., and Gossler, A. (2004). WNT signaling, in synergy with T/TBX6, controls Notch signaling by regulating Dll1 expression in the presomititc mesoderm of mouse embryos. Genes Dev. *18*, 2712–2717.

Holley, S.A., Geisler, R., and Nusslein-Volhard, C. (2000). Control of *her1* expression during zebrafish somitogenesis by a *Delta*-dependent oscillator and an independent wave-front activity. Genes Dev. *14*, 1678–1690.

Holley, S.A., Julich, D., Rauch, G.-J., Geisler, R., and Nusslein-Volhard, C. (2002). *her1* and the *notch* pathway function within the oscillator mechanism that regulates zebrafish somitogenesis. Development *129*, 1175–1183.

Huppert, S.S., Le, A., Schroeter, E.H., Mumm, J.S., Saxena, M.T., Milner, L.A., and Kopan, R. (2000). Embryonic lethality in mice homozygous for a processing-deficient allele of Notch1. Nature *405*, 966–970.

Jiang, Y.J., Aerne, B.L., Smithers, L., Haddon, C., Ish-Horowicz, D., and Lewis, J. (2000). Notch signalling and the synchronization of the somite segmentation clock. Nature *408*, 475–479.

Johnson, J., Rhee, J., Parsons, S.M., Brown, D., Olson, E.N., and Rawls, A. (2001). The anterior/posterior polarity of somites is disrupted in paraxis-deficient mice. Dev. Biol. *229*, 176–187.

Jouve, C., Palmeirim, I., Henrique, D., Beckers, J., Gossler, A., Ish-Horowicz, D., and Pourquie, O. (2000). Notch signalling is required for cyclic expression of the hairy-like gene *HES1* in the presomitic mesoderm. Development *127*, 1421–1429.

Kang, D., Soriano, S., Xia, X., Eberhart, C., De Strooper, B., Zheng, H., and Koo, E. (2002). Presenilin couples the paired phosphorylation of beta-catenin independent of axin. Implications for beta-catenin activation in tumorigenesis. Cell *110*, 751–762.

Koizumi, K.-i., Nakajima, M., Yuasa, S., Saga, Y., Sakai, T., Kuriyama, T., Shirasawa, T., and Koseki, H. (2001). The role of Presenilin 1 during somite segmentation. Development *128*, 1391–1402.

Krebs, L.T., Xue, Y., Norton, C.R., Sundberg, J.P., Beatus, P., Lendahl, U., Joutel, A., and Gridley, T. (2003). Characterization of Notch3-deficient mice: normal embryonic development and absence of genetic interactions with a Notch1 mutation. Genesis *37*, 139–143.

Kulesa, P.M., and Fraser, S.E. (2002). Cell dynamics during somite boundary formation revealed by time-lapse analysis. Science *298*, 991–995.

Leimeister, C., Dale, K., Fischer, A., Klamt, B., Hrabe de Angelis, M., Radtke, F., McGrew, M.J., Pourquie, O., and Gessler, M. (2000). Oscillating expression of c-Hey2 in the presomitic mesoderm suggests that the segmentation clock may use combinatorial signaling through multiple interacting bHLH factors. Dev. Biol. *227*, 91–103.

Lemberg, M.K., and Martoglio, B. (2002). Requirements for signal peptide peptidase-catalyzed intramembrane proteolysis. Mol. Cell *10*, 735–744.

Lewis, J. (2003). Autoinhibition with transcriptional delay: a simple mechanism for the zebrafish somitogenesis oscillator. Curr. Biol. *13*, 1398–1408.

Li, J., Fiei, G.J., Mao, C.-A., Myers, R.L., Shuang, R., Donoho, G.P., Pauley, A.M., Himes, C.S., Qin, W., Kola, I., et al. (2003a). Positive and negative regulation of the γ-secretase activity by nicastrin in a murine model. J. Biol. Chem. *278*, 33445–33449.

Li, T., Ma, G., Cai, H., Price, D.L., and Wong, P.C. (2003b). Nicastrin is required for assembly of presenilin/γ-secretase complexes to mediate Notch signaling and for processing and trafficking of β-amyloid precursor protein in mammals. J. Neurosci. *23*, 3272–3277.

Ma, G., Li, T., Price, D.L., and Wong, P.C. (2005). APH-1a is the principal mammalian APH-1 isoform present in gamma-secretase complexes during embryonic development. J. Neurosci. *25*, 192–198.

Maroto, M., and Pourquie, O. (2001). A molecular clock involved in somite segmentation. Curr. Top. Dev. Biol. *51*, 221–248.

Martoglio, B., and Golde, T.E. (2003). Intramembrane-cleaving aspartic proteases and disease: presenilins, signal peptide peptidase and their homologs. Hum. Mol. Genet. *12*, R201–R206.

Meinhardt, H. (1986). Models of segmentation (New York: Plenum Press).

Minoguchi, S., Taniguchi, Y., Kato, H., Okazaki, T., Strobl, L.J., Zimber-Strobl, U., Bornkamm, G.W., and Honjo, T. (1997). RBP-L, a transcription factor related to RBP-Jk. Mol. Cell. Biol. *17*, 2679–2687.

Neidhardt, L.M., Kispert, A., and Herrmann, B.G. (1997). A mouse gene of the paired-related homeobox class expressed in the caudal somite compartment and in the developing vertebral column, kidney and nervous system. Dev. Genes Evol. *207*, 330–339.

Nomura-Kitabayashi, A., Takahashi, Y., Kitajima, S., Inoue, T., Takeda, H., and Saga, Y. (2002). Hypomorphic *Mesp* allele distinguishes establishment of rostrocaudal polarity and segment border formation in somitogenesis. Development *129*, 2473–2481.

Nyborg, A.C., Jansen, K., Ladd, T.B., Fauq, A., and Golde, T.E. (2004). An Spp reporter activity assay based on the cleavage of a Type II membrane protein substrates provides further evidence for an inverted orientation of the SPP active site relative to presenilin. J. Biol. Chem. *279*, 43148–43156.

Oka, C., Nakano, T., Wakeham, A., de la Pompa, J.L., Mori, C., Sakai, T., Okazaki, S., Kawaichi, M., Shiota, K., Mak, T.W., and Honjo, T. (1995). Disruption of the mouse *RBP-Jk* gene results in early embryonic death. Development *121*, 3291–3301.

Pan, Y., Lin, M.-H., Tian, X., Cheng, H.-T., Gridley, T., Shen, J., and Kopan, R. (2004). γ-secretase functions through Notch signaling to maintain skin appendages but is not required for their patterning or initial morphogenesis. Dev. Cell *7*, 731–743.

Ponting, C.P., Hutton, M., Nyborg, A., Baker, M., Jansen, K., and Golde, T.E. (2002). Identification of a novel family of presenilin homologues. Hum. Mol. Genet. *11*, 1037–1044.

Pourquie, O. (2001). Vertebrate somitogenesis. Annu. Rev. Cell Dev. Biol. *17*, 311–350.

Pourquie, O. (2003). The segmentation clock: converting embryonic time into spatial pattern. Science *301*, 328–330.

Pourquie, O. (2004). The chick embryo: a leading model in somitogenesis studies. Mech. Dev. *121*, 1069–1079.

Rida, P.C.G., Minh, N.L., and Jiang, Y.-J. (2004). A Notch feeling of somite segmentation and beyond. Dev. Biol. *265*, 2–22.

Rozmahel, R., Huang, J., Chen, F., Liang, Y., Nguyen, V., Ikeda, M., Levesque, G., Yu, G., Nishimura, M., Mathews, P.M., et al. (2002a). Normal brain development in PS1 hypomorphic mice with markedly reduced γ-secretase cleavage of βAPP. Neurobiol. Aging *23*, 187–194.

Rozmahel, R., Mount, H.T.J., Chen, F., Nguyen, V., Huang, J., Erdebil, S., Liauw, J., Yu, G., Hasegawa, H., Gu, Y., et al. (2002b). Alleles at the nicastrin locus modify presenilin 1-deficiency phenotype. Proc. Natl. Acad. Sci. USA *99*, 14452–14457.

Saga, Y., and Takeda, H. (2001). The making of the somite: molecular events in vertebrate segmentation. Nat. Rev. Genet. *2*, 835–845.

Sato, Y., Yasuda, K., and Takahashi, Y. (2002). Morphological boundary forms by a novel inductive event mediated by Lunatic fringe and Notch during somitic segmentation. Development *129*, 3633–3644.

Sawada, A., Fritz, A., Jiang, Y.-J., Yamamoto, A., Yamasu, K., Kuroiwa, A., Saga, Y., and Takeda, H. (2000). Zebrafish *Mesp* family genes, *mesp-a* and *mesp-b* are segmentally expressed in the presomitic mesoderm, and Mesp-b confers the anterior identity to the developing somites. Development *127*, 1691–1702.

Saxena, M.T., Schroeter, E.H., Mumm, J.S., and Kopan, R. (2001). Murine Notch homologs (N1–4) undergo presenilin-dependent proteolysis. J. Biol. Chem. *276*, 40268–40273.

Schroeter, E.H., Kisslinger, J.A., and Kopan, R. (1998). Notch-1 signalling requires ligand-induced proteolytic release of intracellular domain. Nature *393*, 382–386.

Schroeter, E.H., Ilagan, M.X.G., Brunkan, A.L., Hecimovic, S., Li, Y.-m., Xu, M., Lewis, H.D., Saxena, M.T., De Strooper, B., Coonrod, A., et al. (2003). A presenilin dimer at the core of the {gamma}-secretase enzyme: insights from parallel analysis of Notch 1 and APP proteolysis. Proc. Natl. Acad. Sci. USA *100*, 13075–13080.

Serneels, L., Dejaegere, T., Craessaerts, K., Horre, K., Jorissen, E., Tousseyn, T., Hibert, S., Coolen, M., Martens, G., Zwijsen, A., et al. (2005). Differential contribution of the three Aph1 genes to γ-secretase activity *in vivo*. Proc. Natl. Acad. Sci. USA *102*, 1719–1724.

Serth, K., Schuster-Gossler, K., Cordes, R., and Gossler, A. (2003). Transcriptional oscillation of Lunatic fringe is essential for somitogenesis. Genes Dev. *17*, 912–925.

Shen, J., Bronson, R.T., Chen, D.G., Xia, W., Selkoe, D.J., and Tonegawa, S. (1997). Skeletal and CNS defects in presenilin-1-deficient mice. Cell *89*, 629–639.

Soriano, S., Kang, D.E., Fu, M.F., Pestell, R., Chevallier, N., Zheng, H., and Koo, E.H. (2001). Presenilin 1 negatively regulates beta-catenin/T cell factor/lymphoid enhancer factor-1 signaling independently of beta-amyloid precursor protein and Notch processing. J. Cell Biol. *152*, 785–794.

Steiner, H., Duff, K., Capell, A., Romig, H., Grim, M.G., Lincoln, S., Hardy, J., Yu, X., Picciano, M., Fechteler, K., et al. (1999). A loss of function mutation of presenilin-2 interferes with amyloid beta-peptide production and Notch signalling. J. Biol. Chem. *274*, 28669–28673.

Sun, X., Lewandoski, M., Meyers, E.N., Liu, Y.H., Maxson, R.E., Jr., and Martin, G.R. (2000). Conditional inactivation of Fgf4 reveals complexity of signalling during limb bud development. Nat. Genet. *25*, 83–86.

Swiatek, P.J., Lindsell, C.E., del Amo, F.F., Weinmaster, G., and Gridley, T. (1994). *Notch1* is essential for postimplantation development in mice. Genes Dev. *8*, 707–719.

Takada, S., Stark, K.L., Shea, M.J., Vassileva, G., McMahon, J.A., and McMahon, A.P. (1994). Wnt-3a regulates somite and tailbud formation in the mouse embryo. Genes Dev. *8*, 174–189.

Takahashi, Y., Koizumi, K.-i., Takagi, A., Kitajima, S., Inoue, T., Koseki, H., and Saga, Y. (2000). Mesp2 initiates somite segmentation through the Notch signalling pathway. Nat. Genet. *25*, 390–396.

Takahashi, Y., Inoue, T., Gossler, A., and Saga, Y. (2003). Feedback loops comprising Dll1, Dll3 and Mesp2, and differential involvement

of Psen1 are essential for rostrocaudal patterning of somites. Development *130*, 4259–4268.

Takasugi, N., Tomita, T., Hayashi, I., Tsuruoka, M., Niimura, M., Takahashi, Y., Thinakaran, G., and Iwatsubo, T. (2003). The role of presenilin cofactors in the gamma-secretase complex. Nature *422*, 438–441.

Taniguchi, Y., Karlstrom, H., Lundkvist, J., Mizutani, T., Otaka, A., Vesting, M., Berstein, A., Donoviel, D., Lendahl, U., and Honjo, T. (2002). Notch receptor cleavage depends on but is not directly executed by presenilins. Proc. Natl. Acad. Sci. USA *99*, 4014–4019.

Urban, S., and Freeman, M. (2003). Substrate specificity of rhomboid intramembrane proteases is governed by helix-breaking residues in the substrate transmembrane domain. Mol. Cell *11*, 1425–1434.

Vincent, S.D., and Robertson, E.J. (2003). Highly efficient transgene-independent recombination directed by a maternally derived *Sox2Cre* transgene. Genesis *37*, 54–56.

Waltzer, L., Bourillot, P.Y., Sergeant, A., and Manet, E. (1995). RBP-Jk repression activity is mediated by a co-repressor and antagolized by the Epstein-Barr virus transcription factor EBNA2. Nucleic Acids Res. *23*, 4939–4945.

Weihofen, A., and Martoglio, B. (2003). Intramembrane-cleaving proteases: controlled liberation of proteins and bioactive peptides. Trends Cell Biol. *13*, 71–78.

Weihofen, A., Binns, K., Lemberg, M.K., Ashman, K., and Martoglio, B. (2002). Identification of signal peptide peptidase, a presenilin-type aspartic protease. Science *296*, 2215–2218.

Weihofen, A., Lemberg, M.K., Friedmann, E., Rueeger, H., Schmitz, A., Paganetti, P., Rovelli, G., and Martoglio, B. (2003). Targeting presenilin-type aspartic protease signal peptide peptidase with gamma-secretase inhibitors. J. Biol. Chem. *278*, 16528–16533.

Wilson, C.A., Doms, R.W., and Lee, V.M.-Y. (2003). Distinct presenilin-dependent and presenilin-independent γ-secretases are responsible for total cellular Aβ production. J. Neurosci. Res. *74*, 361–369.

Wong, P.C., Zheng, H., Chen, H., Becher, M.W., Sirinathsinghji, D.J.S., Trumbauer, M.E., Chen, H.Y., Price, D.L., Ploeg, L.H.T.V., and Sisodia, S.S. (1997). Presenilin 1 is required for *Notch1* and *Dll1* expression in the paraxial mesoderm. Nature *387*, 288–291.

Wrigley, J.D., Schurov, I., Nunn, E.J., Martin, A.C., Clarke, E.E., Ellis, S., Bonnert, T.P., Shearman, M.S., and Beher, D. (2004). Functional overexpression of gamma-secretase reveals protease independent trafficking functions and a critical role of lipids for protease activity. J. Biol. Chem. *280*, 12523–12535.

Yang, X., Klein, R., Tian, X., Cheng, H.-T., Kopan, R., and Shen, J. (2004). Notch activation induces apoptosis in neural progenitor cells through a p53-dependent pathway. Dev. Biol. *269*, 81–94.

Zhang, Z., Nadeau, P., Song, W., Donoviel, D., Yuan, M., Berstein, A., and Yankner, B.A. (2000). Presenilins are required for γ-secretase cleavage of β-APP and transmemebrane cleavage of Notch-1. Nat. Cell Biol. *2*, 463–465.

Zhang, N., Norton, C.R., and Gridley, T. (2002). Segmentation defects of Notch pathway mutants and absence of a synergistic phenotype in lunatic fringe/radical fringe double mutant mice. Genesis *33*, 21–28.